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PRINCIPAL INVESTIGATOR: Sharon Y. Roth, Ph.D.

CONTRACTING ORGANIZATION: University of Texas M.D. Anderson Cancer Center
Houston, Texas 77030

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Sharon Y. Roth, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Texas M.D. Anderson
Cancer Center
Houston, Texas 77030
E-Mail: syr@mdacc.tmc.edu

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13. ABSTRACT (Maximum 200 Words)

Nucleosomes are folded together into chromatin structures that inhibit transcription. Studies here test the idea that an enzyme, Gcn5, that regulates chromatin folding is important for p53 functions and for estrogen responses. Experiments were proposed to 1) determine whether Gcn5 serves as a coactivator for activation of gene expression by the estrogen receptor 2) examine biochemical, molecular, and genetic connections between Gcn5 and p53 and 3) to generate a mammary gland specific 'knock out' of Gcn5 in mice to create a mouse model for Gcn5 functions in breast development and tumor formation. We have made good progress towards all three aims. We have examined Gcn5-dependence of estrogen responses in ER+ cell lines. We have generated mice that carry null alleles for both Gcn5 and p53 in cis on chromosome 11. We have generated mice that carry a conditional disruption allele for Gcn5 that will allow us to do a breast-specific knock out in the coming year. Our studies will provide new information about breast cancer biology. Moreover, they will allow us to determine whether histone acetyltransferases might provide targets for development of new drug therapies or diagnostic agents, furthering our advancement towards eradication of this disease.

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INTRODUCTION

The compaction of DNA into chromatin in the eukaryotic nucleus regulates gene expression by controlling access of basal transcription proteins and regulatory factors (Edmondson and Roth, 1996). Two major classes of chromatin remodeling activities have been identified to date (Struhl, 1998; Wade and Wolffe, 1999). One class includes large ATP-driven complexes typified by Swi/Snf, which regulate nucleosome placement and movement. The second class is comprised of enzymes responsible for regulating post-translational modifications of the histone proteins. Of these, histone acetyltransferases (HATs) and histone deacetylases (HDACs) are best characterized (Roth et al., 2001). Specific HATs have been implicated in cancers, including breast cancer (Anzick et al., 1997). HATs also act as cofactors for tumor suppressors such as p53 or for hormonal responses (Blanco et al., 1998; Liu et al., 1999; Schiltz and Nakatani, 2000). The GCN5 HAT is highly related to PCAF (Xu et al., 1998), which has been linked both to p53 and estrogen receptor functions. Experiments supported by this grant were designed to determine the role of GCN5 in these processes and in normal breast development. The proposal had three specific aims: 1) to determine whether GCN5 serves as a coactivator for the estrogen receptor 2) to examine biochemical, molecular and genetic connections between GCN5 and p53 and 3) to generate a mammary gland specific 'knock out' of GCN5 in mice.

BODY

Our Statement of Work was organized into three main tasks. We have made good progress on each of these tasks as described below:

Task1: To determine whether GCN5 serves as a coactivator for the estrogen receptor in transcriptional activation in mammalian cells.

- Goal 1: Transfect NIH 3T3 cells with expression constructs for the estrogen receptor (ER), FLAG-tagged GCN5, and an estrogen responsive reporter gene. Monitor expression of the reporter gene in the presence and absence of estradiol.

  Progress: As reported last year, this task was completed by the end of the second funding period. Unfortunately, the results of these transient transfection experiments were inconclusive and suggested to us that we needed to create stable cell lines carrying estrogen responsive reporter genes integrated into the chromosome and that we needed to examine additional types of cells. To this end, we selected stable cell lines (MCF7 and CV1 cells) that carry an integrated reporter gene and but these cells were unhealthy and were not useful for our studies. Therefore, we transfected GCN5 expression constructs into breast cancer cell lines that express the ER. GCN5 protein was not stable in these cells, again thwarting our efforts to examine its role in estrogen responses. Because the mouse experiments described in Task 3 will be more definitive than these cell culture experiments, priority was shifted towards that task.

- Goal 2: Construct mutated forms of GCN5 for transfection experiments to determine the domains required for estrogen mediated functions.

  Progress: As reported last year, we constructed point mutations in the GCN5 HAT catalytic center. Although we originally planned to test these in cell culture experiments, we decided to test them in mice, given the problems encountered above. To this end, we created targeting alleles to replace the wild type Gcn5 gene with these mutated alleles. We now have chimeric mice that carry the mutated alleles and are breeding these to generate mice that are heterozygous for the mutated alleles. These mice will then be intercrossed to generate mice homozygous for the mutant alleles. This work should be completed in the coming year.

- Goal 3: Perform in vitro binding experiments to determine if GCN5 interacts directly with the estrogen receptor.

  Progress: These experiments were placed on hold due to our inability to demonstrate a role for Gcn5 in estrogen responses in tissue culture cells (Task 1). If the experiments in Task 3 indicate that GCN5 is important for estrogen responses, we will reinitiate these in vitro binding experiments.

- Goal 4: Perform co-immunoprecipitation experiments to determine if GCN5 is associated (directly or indirectly) with the estrogen receptor in vivo.

  Progress: As above, these experiments are on hold pending the outcome of the experiments in Task 3.

Task 2: To examine the role of GCN5 in p53 functions.

- Goal 1: Express and purify recombinant p53.

  Progress: As reported previously, this task was completed.

- Goal 2: Test recombinant p53 as a substrate for recombinant GCN5.

  Progress: Completed. We found that GCN5 can acetylate p53 in vitro.

- Goal 3: Perform co-transfection experiments to determine if GCN5 augments p53 transactivation in vivo, and if so, determine which domains in GCN5 are required for this effect.

  Progress: Completed. Unfortunately, we could never demonstrate any augmentation of p53 responses upon cotransfection with GCN5 in transient transfection experiments (see Fig. 2 for example). This lack of response may indicate that GCN5 functions may only be required when the reporter gene is packaged into chromatin. Therefore we examined expression of the endogenous p21 gene, which is p53 responsive. Again we saw no effect of addition of exogenous GCN5 to the cells (data not shown). We would like to try these experiments again in cell lines that lack endogenous GCN5, since endogenous levels of this enzyme may obscure any effects of the transfected GCN5. We have recently generated GCN5 null (mouse) embryonic stem cells, and hope that we can use a conditional allele of Gcn5 to generate additional GCN5 null cell lines in the future. If so, we will revisit these experiments. We may also use our catalytic site GCN5 mutants generated above in Task1 (goal 2) as 'dominant negative' forms of GCN5 to determine whether Gcn5 is involved in p53 functions.

- Goal 4: Cross mice heterozygous for the GCN5 null allele with mice homozygous for the p53 allele.

  Progress: Completed. We have successfully generated mice that are null for p53 and heterozygous for the GCN5 null allele. We are continuing our breeding to generate more of these mice, as well as mice that heterozygous for both the p53 null allele and the Gcn5 null allele.

- Goal 5: Cross offspring from the above matings to generate mice that are null for both p53 and GCN5, to see if loss of p53 rescues the embryonic lethality resulting from GCN5 loss.

Progress: Nearly completed. Gcn5 and p53 are both encoded on chromosome 11, so we had to isolate mice that had undergone a recombination event to place both mull alleles on the same copy of this chromosome. We were able to intercross these mice to generate the double mutants. The loss of p53 did not rescue the lethality of the GCN5 null mice. However, the double null embryos are slightly more developed than the GCN5 null embryos, indicating that p53 does contribute to part of the GCN5 null phenotype.

Task 3: To generate a mammary gland-specific 'knock out' of GCN5 in mice.

- Goal 1: Construct targeting vector for GCN5 gene replacement.

  Progress: Completed.

- Goal 2: Transform targeting vector in ES cells and select/screen properly targeted cells.

  Progress: Completed. We identified 7 independent clones that carry the properly targeted replacement allele (Fig. 3, for example).

- Goal 3: Inject above ES cells into blastocysts to generate chimeric mice.

  Progress: Completed.

- Goal 4: Breed chimeric mice with wild type mice to generate mice heterozygous for the replacement allele.

  Progress: Completed.

- Goal 5: Intercross heterozygous mice to generate mice homozygous for the replacement allele.

  Progress: In progress. Unfortunately, our first chimeras (reported last year) never passed the GCN5 'floxed' allele to their offspring during several months of breeding these mice. Therefore, we generated new chimeras during the last six months and are now breeding these.

- Goal 6: Breed homozygous mice with WAP-cre transgenic mice.

  Progress: Pending. We cannot begin these breedings until the above mice are generated.

- Goal 7: Analyze GCN5 removal and mammary gland development and mammary tumor formation in female mice resulting from the above cross before, during, and after pregnancy.

  Progress: Pending. We cannot do this analysis until goal 6 has been met. However, we anticipate that these experiments will be completed in the next 12-18 months.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstration that GCN5 can acetylate p53 in vitro.
- Creation of mice that carry null alleles of p53 and Gcn5 in cis on chromosome 11, and characterization of double mutant phenotype.
- Creation of GCN5 catalytic site mutants and chimeric mice carrying these mutations.
- Construction of a floxed allele of GCN5 and creation of mice that carry this allele.

REPORTABLE OUTCOMES

Publications: Our work should be ready for publication in the coming year. We will publish one paper on the genetic interactions between GCN5 and p53. Another paper will report the phenotypes of the mice carrying the GCN5 catalytic site mutations. A third paper will describe the function of GCN5 in normal breast development in the mouse, and the effects of eliminating GCN5 in breast tissue.

Abstract:

Degrees Obtained: Huy Phan completed the PhD portion of his Md/PhD degree in June 2002. His thesis project was supported in part by this grant. Yvonne Evrard will complete her Ph.D. thesis project within the next year. Part of Yvonne's stipend was paid from this grant and much of her research was supported by this grant.

Funding Obtained: Based in part on some of the preliminary data and mouse models developed in this project, I was recently awarded an RO1 grant from the NIH to study the functions of GCN5 and other histone acetyltransferases during mammalian development.

CONCLUSIONS:

We have determined that transient transfection experiments are limited in their ability to delineate the role of GCN5 as a coactivator in p53 or ER responses. Therefore our later studies focused on the mouse models created in Tasks 1, 2, and 3. In the long term, our studies may reveal new ways of diagnosing breast tumors or new strategies for treatment. In addition, these studies will provide novel insights into the role of chromatin modifying activities in normal breast development and in tumor formation.

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Xu, W., Edmondson, D. G., and Roth, S. Y. (1998). Mammalian GCN5 and P/CAF acetyltransferases share homologous amino-terminal domains important for the recognition of nucleosomal substrates, Mol Cell Biol *18*, 5659-5669.

FINAL REPORT SUMMARY

Bibliography: No papers have been published yet, but the following manuscripts will be submitted in the near future:

1. Evrard, Y., Bu, Ping, and Dent, S.Y.R. Loss of p53 allows a partial rescue of embryonic defects in GCN5 null mice.
2. Evrard, Y. and Dent, S.Y.R. Genomic instability associated with GCN5 loss in early mouse embryos.

Meeting Abstract:

Wyszomierski, S. and Dent, S.Y.R. PCAF and GCN5 in mouse mammary gland development, presented at the DOD USAMRMC Era of Hope meeting, 2002.

Personnel who received pay from this grant:

Sharon R. Dent
Diane Edmondson
Yvonne Evrard
Vijaya Vuyyuru

APPENDIX

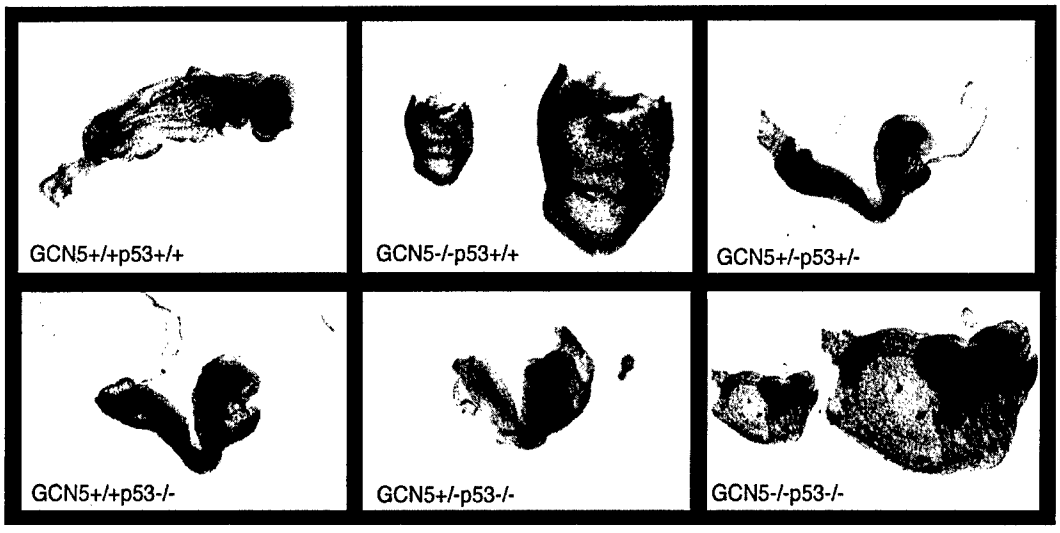

Fig 1. Loss of p53 partially rescues the embryonic abnormalities of GCN5 null embryos. Whole embryos with the indicated phenotypes are shown. Notice that the p53-/- GCN5-/- double mutants are more developed than are the GCN5 -/- embryos but are less developed that the p53-/- embryos.

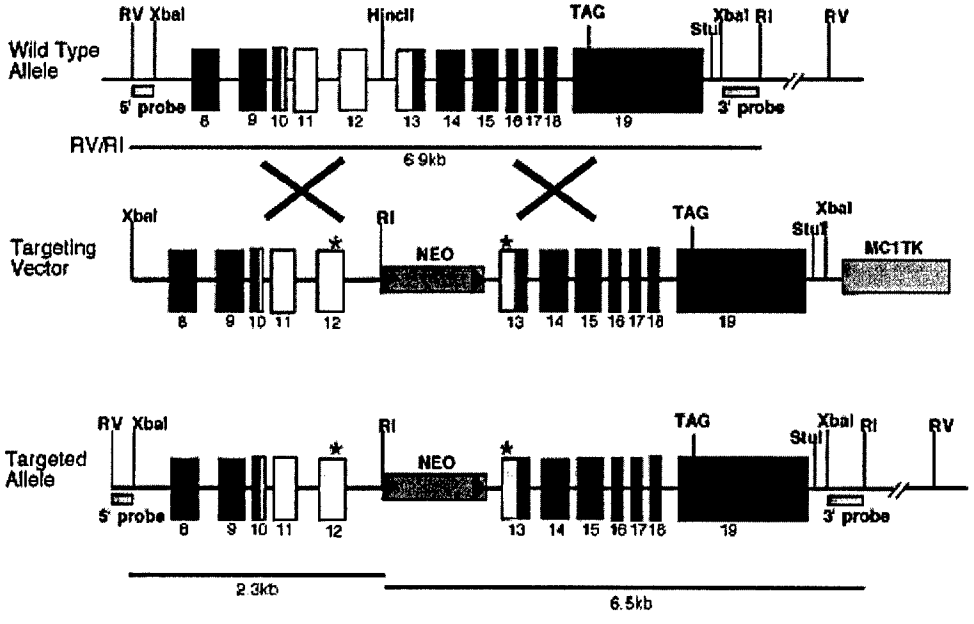

Fig.2. Targeting strategy to introduce point mutations into Gcn5 genomic sequences. The positions of the mutations in the catalytic domain encoded in exons 12 and 13 are indicated by asterisks. For convenience, all three mutations are shown here as part of the same vector, but different versions of this vector containing separate mutations were actually be used. Homologous recombination events were first detected by Southern blot of Eco RI-EcoRV digested genomic DNA using the 5' and 3' probes indicated. The presence of the mutations were then confirmed by PCR of a fragment spanning the mutation site and restriction digestion. A PvuI site accompanies the E568A mutation, PstI accompanies D608A, and StuI accompanies G582/584A mutations.